Simultaneous removal of Sulphate and Chromium from Tannery Waste using Microbes

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CERTIFICATE

*This is to certify that the thesis entitled, "**Simultaneous removal of Chromium and Sulphate from Tannery waste using microbes**" submitted by **Ms. Urmi Mullick** (210CH1040) for the award of Master of Technology Degree is a record of bonafide research carried out by her at the Chemical Engineering department, National Institute of Technology, Rourkela under my supervision and guidance. The work documented in this thesis has not been submitted to any other University or Institute for award of any other degree.*

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ABSTRACT

Leather industry contributes to one of the major industrial pollution problems our country is facing today. Microbes (bacteria/fungi) are the most important eco-friendly agents for the degradation and detoxification of industrial pollutants along with organic waste removal. Extensive research has been carried out to find suitable, resistant and efficient microbes to treat the tannery effluent. In the concerned study, we have tried to isolate the organisms from the tannary effluent acclimatized them to increasing Chromium and Sulphate rich environment which may later be utilized towards bioremediation of both the pollutants. In this direction, bacteria resisting 240mg/l Cr and 280mg/l and others with 170mg/l Cr and 200mg/l sulphates have been obtained and tested for growth rates and Cr removal capacity. A maximum of 99.8% Cr removal have been achieved in four days of submerged culture by *Micrococcus yunnanensis*. The consortium of *Pseudomonas aeruginosa* & *Micrococcus yunnanensis* microbes is found to be best performing interms of biomass growth and removal of Cr than the individual isolate. These two microbes also remediated about 24% sulphate from the culture. The optimal parameters for treatment process were pH 7, temperature 35^{o}C, rpm 100 and Cr concentration 150 mg/l. Laboratory scale fermenter study maintaining the optimized parameters showed 97.5 % removal of Cr in just 48 hours and the highest of which is 98% achieved in 56 hours. In a second step, the effluent was treated by a consortium of SRBs (*Desulfovibrio desulfuricans, D. vulgaris* and *D. gigas*) that utilize Cr(VI) as an electron acceptor for sulphate reduction and hence both Cr(VI) and sulphates get removed from the broth. From the initial concentrations of 150mg/l and 200 mg/l of Cr(VI) and sulphate in the simulated wastewater, it was brought down to 0.1 mg/l and 70.2 mg/l for Cr and sulphate respectively. Hence in a two stage biological treatment process for tannery waste, an excellent 99.9% Chromium and 63.9 % sulphate removal was achieved.

Chapter 1

INTRODUCTION

Industrial growth not only offers a better lifestyle to human beings but also provides employment opportunity to millions of people worldwide. Perfectly finished footwear, bags, wallets, covers *etc*. made up of skin of different animals is accepted and used all over the world. This sets up a huge leather industry with billions of turn-over every year. The major global players in this sector now are China, Italy, India, Brazil, Taiwan, Korea and Vietnam where India grabs the second largest share of global market next to China.

Leather industry in India, is one of the greatest contributors towards the economy of the nation as it is one of the oldest and most practiced manufacturing industries. In India, thousands of industrial tannery units are spread mostly across Tamil Nadu, West Bengal, Uttar Pradesh, Andhra Pradesh, Karnataka, Maharashtra, Rajasthan and Punjab.

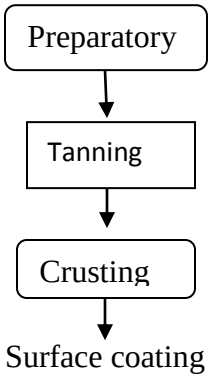
Over half of India's leather manufacturing units are built around Ganga river basin (web. ref. 4). West Bengal alone 600 tanneries are functioning employing and 20,000 units manufacturing leather products providing employment to more than 200,000 people. Kanpur, which is otherwise known as the 'Leather City of the World' has over 1600 functional leather manufacturing units producing semi-finished, finished and value-added products (web. ref. 2).

As per an investigation by the Inter Ministerial Group, India's leather export should approach the US $ 7 billion mark present year. The biggest importers of leather are the US, Germany, France to name a few. The industry is showing a massive potential and competitive growth of offering more exports, investment scope and employment opportunities in the near future.

1.1 Process in brief:

The raw hide/skin has to undergo a series of physical and chemical treatments (Table 1) before it turns into usable flattering leather. The principal leather making protein, collagen, exists in association with various globular proteins, viz. albumin, mucoids, globulin,; and fibrous proteins such as elastin, keratin, and reticulin in hides and skins. During leather manufacturing, the noncollagenous constituents are partially or completely removed in the various pre-tanning operations. Besides chemical treatment, certain enzymatic treatments are also performed to achieve optimum results [1]**.**

The leather manufacturing process is divided into three sub-processes: preparatory stages, tanning and crusting. The manufacturing process follows these steps:



1.1.1 PREPARATORY STEPS:

The preparatory stages are when the hide/skin is prepared for tanning. Following processes are performed the preparatory stage (web. ref. 1):

- Preservation- the hide is treated to renders it temporarily unputrescible.
- Soaking - water for washing or rehydration is reintroduced to the hides.
- Liming - unwanted proteins are removed.
- Unhairing - the majority of hair from the skin is removed.
- Fleshing - subcutaneous material/flesh is removed.
- Deliming – removal of the liming and unhairing chemicals from the pelt.

- Bating - introduction of proteolytic proteins to the skin to remove unwanted proteins and to assist with softening of the pelt.
- Degreasing - natural fats/oils are stripped or as much as is possible from the hide/skin.
- Bleaching - chemical modification of dark pigments to lighten up the color of the pelt.
- Pickling - lowering the pH to the acidic region. Pickling is normally done to help the penetration of tanning agents such as chromium into the hide.
- Depickling - raising of the pH out to basic region to assist with penetration of certain tanning agents.

1.1.2 TANNING:

Tanning is the process of converting the protein of the raw hide or skin into a flexible and stable material which will not putrefy when wetted back. It protects the leather from environmental effects such as wet heat, moisture, sweat and microbial degradation [2]. A large number of different tanning materials and methods can be used such as salts of Chromium, Aluminum, zirconium and organic tanning agents; the most commonly used tanning material is chromium. Chromium forms cross-links between the collagen fibers as a result the hide gains a good mechanical resistance, an extraordinary dyeing suitability and a better moisture resistance in comparison with hides treated with vegetable substances [3].

1.1.3 CRUSTING:

Crusting is when the hide/skin is thinned, retanned and lubricated. Crusting may include operations like wetting back, splitting the hide, neutralizing acid/base, retanning, dyeing, stripping, conditioning etc. It is the final process which leads to finishing operations of a flattering leather product.

(Table 1: Chemicals used in leather making process)

CHEMICAL NAME	**USES**
Biocides	Prevents the growth of bacteria on hides
Degreasers	Removal of oil and fats
Lime	Swell the hides
Sodium Sulphide, Sodium Hydrosulphide	Chemically destroys the hair
Caustic soda	Liming process
Ammonium Sulphate	Deliming process
Sodium Metabisulphite	Deliming, bleaching
Formic acid, Sulphuric acid	Lowering the pH during pickling
Salt	Prevents the acid swelling during pickling
Sodium Formate	Helps penetration of Chromium tanning salts
Chromium Sulphate	Tanning agent
Aldehydes	Tanning agents to make wet-whites
Magnesium Oxides	Basification process to raise the pH
Sodium Formate, Sodium Bicarbonate	Raise pH during neutralization
Formic Acid	Lowers the pH during retanning
Chrome syntans , Chromium Suphate	Rechroming to improve the texture
Resins, Polymers	Give fullness and tight grains to leather
Dyes	Coloring agents
Nitrocellulose lacquers ,Polyurethane lacquers, Acrylic lacquers	Top coat of a leather finish

1.2 Health Hazard due to Chromium and Sulfates:

Chromium is an essential trace metal for living organisms [4] but exhibits toxicity even at low concentrations like 20µg/l (EPA, 1998). It is highly soluble and in environment, occurs in two major oxidation states i. e. Cr(III) and Cr(VI).

The fate of chromium inside the body depends on the oxidation state. Chromium(VI) readily penetrates cell membranes whereas chromium(III) does not. Cr(VI) is found to be 1000 times more toxic than Cr(III). Cr(III) in aqueous phase could be oxidized through interaction with compounds like manganese dioxide (MnO_2) to Cr(VI) rendering it hazardous[5,6].

Cr(VI) induces acute and chronic toxicity, neurotoxicity, dermatotoxicity, genotoxicity, carcinogenicity, immunotoxicity, and general environmental pollution [7]. Once transported through the cell membrane, chromium(VI) is rapidly reduced to chromium(III), which subsequently binds to macromolecules. Accumulation of Chromium takes place mainly in liver, kidneys, spleen, and bone marrow after entering the system. Oversensitivity to Cr may cause skin diseases like epidermal dermatitis on little exposure to the element. Also it induces respiratory carcinogenicity in humans exposed to chromium(VI) in occupational settings.

Health hazards due to Sulfate containing water intake are relatively mild. Cathartic effects are commonly experienced by people consuming drinking-water containing sulfate in concentrations exceeding 600 mg/litre (US DHEW, [8]). Children, transients and the elderly are at potentially high risk of dehydration from diarrhoea that may be caused by high levels of sulfate in drinking-water. Generally, laxative effect at concentrations of 1000–1200 mg/litre is observed but no severity is found as body starts adapting to higher sulfate levels. The presence of sulfate also results in a noticeable taste in drinking-water.

1.3 ENVIRONMENTAL FOOTPRINTS:

The processes of leather manufacturing have a high environmental impact, most notably due to the heavy use of toxic chemicals in the tanning process.

About 90% of tanneries in the world use chromium salts as tanning materials because of the excellent properties of the chromium compounds in the tanning [9]. Only a fraction of this salt provides the desired quality to the hide. 60% of this Cr is used up in the tanning process and rest chromium remains in the tanning effluent [14]. In such aqueous waste, Cr(VI) is present as either dichromate ($Cr2O_7^{2-}$) in acidic environments or as chromate (CrO_4^-) in alkaline environments.

However 90% of this salt or Chromium, evacuated from the tanneries as waste product, subsequently finds its way into river and groundwater systems causing pollution and contamination of the later.

One tonne of hide or skin processing generally leads to the production of 20 to 80 m^3 of turbid, toxic and foul-smelling wastewater. Thus the tanning process is responsible for the generation of large quantities of chromium waste, which is also loaded with organic matter , and salts such as chlorides, sulfates and carbonates, as well as ammonia, detergents, emulsifiers, bactericides, fungicides, dyes, skin proteins, hair, fat, and other components [10]. Disposal of this Chromium rich potentially toxic waste in a manner not to cause adverse effects on the surrounding land, water, and the local flora and fauna is an emerging problem for environmental research.

With solid wastes composing up to 70% of the wet weight of the original hides, the tanning process exerts considerable strain on water treatment installations. Due to this, the leather processing industry is one of the worst offenders of the environment. Hence a balance between the environmental measures in place and the opportunity for the leather industry to grow is the need of the time.

1.4 *Organization of the thesis:*

First chapter renders an overview of the leather processing steps, tanning process and the waste generated due to it. This chapter also presents identification of the problem, objectives of the present work with the thesis outline. The second chapter discusses different techniques used so far for tannery waste treatment with the mention of significant previous contributions. In chapter three, the experimental work carried out for the project has been presented. Results and facts supporting the results have been stated in the fourth chapter. In an ending note, the fifth chapter contains the findings of the present work and conclusions with recommendations for future research.

Chapter 2

LITERATURE REVIEW

2.1 WATER QUALITY PARAMETERS:

Water quality parameters are the physical, chemical and biological characteristics of water. A number of parameters determine the quality of water intended for a general or specific use. Pure water is colorless and should have a pH of 6.5 to 8.5. The degree of pollution in water and it's affect can be analyzed by analyzing following parameters.

2.1.1 Dissolve Oxygen (DO):

Adequate dissolved oxygen is considered necessary for good water quality. Oxygen is a necessary element to all life forms. Aquatic life is put under stress if DO levels in water drop below 5.0 mg/l. DO is expressed in mg/l at a particular temperature as it varies with water temperature and altitude.

2.1.2 Chemical Oxygen Demand (COD):

Chemical oxygen demand (COD) test is commonly used to indirectly measure the amount of organic compounds in water. It is expressed in (mg/L), which indicates the mass of oxygen consumed per liter of solution. The dichromate reflux method is preferred for experimental determination of COD and maximum oxygen demand between 250-300 mg/l must be reached before waste water can be returned to the environment.

2.1.3 Biochemical Oxygen Demand (BOD):

Biochemical oxygen demand or BOD is the amount of dissolved oxygen demanded for aerobic organisms in a body of water to oxidize organic material present in a given water sample at certain temperature over a specific time period (web. ref. 5). It gives the idea of biodegradability of any sample and strength of waste also the self-purification capacity of water bodies. Industrial streams with high BOD if channeled to streams may deplete all the dissolved oxygen and pose

risk to aquatic life. In India, it is taken as 3-day BOD at 27^{o}C expressed in mg/l. Water with BOD below 100mg/l qualifies to be channeled to water bodies.

2.1.4 Total Organic Carbon (TOC):

TOC (Total Organic Carbon,) an important sum parameter for assessing the organic load of water. As all organic carbon compounds are determined and specified in terms of carbon mass, TOC is an absolute definable quantity and is directly measurable (unit: mg C/l). Its permissible limit is 4 mg/l.

The organic carbon in water is composed of a variety of organic compounds in different oxidation states. Some of these carbon compounds are oxidizable further by biological or chemical agents or processes, and the biochemical oxygen demand (BOD) and chemical oxygen demand (COD) may be used to characterize these fractions. TOC on its own is not accountable for the oxidisability of the measured total carbon or the amount of oxygen needed for its biodegradation. Low TOC is a confirmation of the absence of potentially harmful organic chemicals in water.

2.1.5 Total Dissolved Solids (TDS):

The dissolved minerals in water are commonly referred to as Total Dissolved Solids (TDS). The TDS content of any water is expressed in mg/l or in parts per million (ppm). The minerals are basically compounds (salts) of carbonates, bicarbonates, chlorides, sulphates, phosphates and nitrates of Calcium(Ca), Magnesium(Mg) and Sodium(Na) and Potassium(K) etc. and a small amount of organic matter and dissolved gases. Water containing TDS concentrations below 1000 mg/l is usually acceptable as per WHO standards.

2.1.6 Electrical Conductivity (EC):

Electrical conductivity is the indication of the total ionized constituents of water. It is relates to the sum of the cations (or anions), and is closely correlated, with the total salt concentration. Electrical conductivity values are always expressed at a standard temperature of 25°C moh/cm. Conductivity of pure water is 0.05µS/cm and hence electrical conductivity of sample indicates the presence of dissolved and dissociated salts.

Nearly 90% of the tanneries in India are engaged in the chrome tanning process. Most of them discharge untreated wastewater into the nearest water bodies. Wastewater discharged from this industry is highly complex, concentrated, and toxic. These pollutants are expressed in terms of chemical oxygen demand (COD), 5-day biochemical oxygen demand (BOD5), total suspended solids (TSS), and total Kjeldahl nitrogen (TKN), as well as sulfur, phosphorus, and chromium compounds.

A review of the technical literature indicates that many processes have been investigated for tannery wastewaters, ranging from the simple to the advanced complicated methods. These methods include physico-chemical treatment, ion exchange, membrane filtration, and electrochemical systems, biological treatment (aerobic and anaerobic treatment).

Esmaeili et al., 2005 reported that chromium precipitation is a relatively simple technique in which chromium and other metals are precipitated as highly insoluble hydroxides [11].

Song et al., 2003 reduced COD and TSS by using aluminum sulfate and ferric chloride as coagulant material with COD and TSS removal efficiencies of 30–37% and 38–46% respectively [12].

Ozone and ultraviolet (UV) radiation technologies are also used to remove pollutants in the tannery effluents. However, the high cost of ozone still remains an important drawback of these processes. Electrochemical oxidation was also used for final tannery wastewater treatment showing complete mineralization of vegetable tannery wastewater (*Panizza et al., 2004*) [13].

Pre-treatment of tannery wastewater using two systems, electrolytic and physico-chemical systems, showed poor efficiencies for the electrolytic system and significant removal of pollutants with the physico-chemical system.

Szpyrkowicz et al. 2005 used a combination of electrochemical and biological processes for tannery wastewater treatment [14].

All these processes become cost ineffective when there is a huge amount of waste water to be treated. Also the sludge produced still contains the toxic materials. Previous studies have shown that microorganisms can reduce Cr(VI) efficiently and could be used to treat Cr(VI) contaminated water under neutral pH conditions. Hence further investigations were carried out biological treatment remains the most suitable process to treat organic waste.

Hypersaline effluents (*i.e.* wastewater containing more than 35 g l^{-1} total dissolved solids) are generated by various industrial activities. Such wastewater, rich in both organic matter and total dissolved solids (TDS), is difficult to treat using conventional biological wastewater treatment processes. High salt content is considered to have an inhibitory effect on anaerobic digestion. It was therefore proven that the adaptation of an active methanogenic biomass to the salinity of the effluent was possible. *Larsen, 1962,* used halophilic bacteria to treat hypersaline effluent [15].

O. Lefebvre et al.,2006 studied the anaerobic digestion of hypersaline tannery soak liquor using an upflow anaerobic sludge blanket (UASB) reactor with an aerobic post treatment and achieved 95% efficiency. When the UASB was followed by an aerobic post treatment, 78% COD removal at an organic loading rate (OLR) of 0.5 kg COD/m3/d, a hydraulic retention time (HRT) of 5 days, and a total dissolved solids (TDS) concentration of 71 g/l. The combination of the UASB followed by an aerobic treatment enhanced the performance of the overall wastewater treatment process and the efficiency of COD removal for the combined anaerobic/aerobic treatment system reached 96% [16, 17].

- However, for effective operation, the system had to be operated at very low Oxygen Loading Rates, which affects the economic viability of such a process.

Mahmoud A. El-Sheikh et al. 2011, studied the advantage of segregating tannery soak liquor from the composite tannery wastewater, in order to treat it separately by anaerobic digestion. The anaerobically pre-treated soak liquor could then be mixed with the composite wastewater to undergo aerobic treatment using two stage upflow anaerobic sludge blanket (UASB) reactor. This raw wastewater has pre-treated through physico-chemical treatment before entering the two stage UASB reactors. The anaerobic digestion of the tannery soak liquor was studied for more than 300 days with varying Hydraulic Retention Time (HRT). Pollutants were expressed as chemical

oxygen demand (COD), 5-day biochemical oxygen demand (BOD_5), total suspended solids (TSS), and total Kjeldahl nitrogen (TKN), as well as sulfur, phosphorus, and Chromium. This makes the treatment process very lengthy and cumbersome. The most of COD and BOD5 removal occurred in the first stage UASB1 with COD removal through all operational phases were 78.2% and 21.8% and the mean values of BOD5 along all operational phases were 83% and 17% for UASB1 and UASB2 respectively [18].

- Hence this process demands a pre-investigation of the quality of the raw wastewater to be treated and the overall required treatment capacity prior to discharge as to justify the use of the second UASB reactor. This limits its application.

Song et al. 2003, also developed an up-flow anaerobic fixed biofilm reactor (UAFBR) to treat tannery Waste water and obtained good COD and TSS removals even under conditions of temperature shock [12].

Tannery wastewater treatment is affected by the presence of substances inhibiting nitrification and denitrification, the need for addition of an external readily biodegradable COD source to sustain denitrification, and a high COD effluent at the end of the process due to the presence of organic biorefractory and inhibiting compounds. These difficulties are usually overcome by adopting completely mixed reactors, low organic loads and chemical–physical pre-treatments with a big increase in treatment costs.

G. Farabegoli et al, *2004*, carried out experiments to determine the feasibility and efficiency of treating tannery wastewater containing chromium, with sequencing batch reactors (SBR). They selected and enriched particular microbial species tolerating upto 180 mg/l Cr inside the reactor and more capable of carrying out biological processes such as nitrification and denitrification in the presence of inhibiting substances and evaluated parameters (COD, TKN, NOx-N, SSV) were determined to analyze the performance of the SBR fed with untreated tannery wastewater containing a relevant amount of chromium. Following this way it was possible to achieve economic savings, due to the absence of the chemical–physical treatment, and to treat higher COD loads avoiding the addition of an external carbon source to sustain denitrification. SRB

offered selection and enrichment of particular microbial species more capable of carrying out the biological processes [19].

Biological sulphate reduction (BSR) has been cited as a method for the treatment of sulphate and metal-rich waters originating from the mining industry. A problem associated with the treatment of acid mine drainage (AMD) by BSR is the lack of an electron donor for the SRBs. Tanneries produce effluents that can be used as a C-source for these treatment plants.

G. Boshoff et al., 2004 ,blended of tannery effluent with acid mine drainage effluent that not only negated the need to provide a carbon source for biological treatment, but allowed for the simultaneous treatment of both acid mine drainage and tannery effluent [20].

- The proximity of a tannery to a mine's AMD source and the amount of tannery effluent available determines the feasibility of its use.

Chromium(III) is used in tannery as chromium sulphate, which may be converted into chromium(VI) effluent. Hexavalent chromium converts to Chromium(III) which is much less toxic and less soluble by several microorganism possess chromate reductase. Microbial viability is essential for biotransformation as these reactions are enzyme mediated. The use of microbial cells as biosorbents of heavy metals is a potential alternative to conventional methods used to decontaminate liquid wastes.

Micera and Dessi, 1988, [21]used bacterial strain, *Acinetobacter sp*., for removal of chromium from tannery effluent in sequential bioreactor indicated 80% reduction in chromium after 15 days (Shrivastava and Thakur, 2003) [22].

- But such a long time for removal of chromium may lead to generation and accumulation of metal and toxic compounds in the environment. Effective and efficient removal of chromium in short time is basic necessity of present time.

Shaili Srivastava et al, 2006, investigated fungal strains isolated from tannery effluent and are optimized process parameters in presence of toxic form of chromium [Cr(VI)] for removal of chromium from tannery effluent and soil. Sucrose, dextrose, sodium acetate and sodium citrate

were used for optimization of carbon source with pH 6.0 and obtained 78% chromium reduction at temperature 30^{o}C by the strain FK1. Uptake of chromate was 8.2 mg/gm dry weight of mycelium. They used gravel in the bioreactor in order to immobilize the biomass. Most of the fungi have no potential to degrade chlorinated phenolic compounds, but they absorb chromium. However, bacteria degrade chlorinated compounds, but they cannot absorb higher concentration of chromium [23].

A.Sathiavelu et al., 2010,employed strains of *Pseudomonas sp.* and achieved *95-99%* reduction of Cr in waste water [24]. They isolated bacteria that could tolerate up to 1000µg/ml of K_2Cr2O_7

M. Vainshtein et al., 2003, described Cr (VI)-reduction by a bacterial consortium consisting of anaerobic SRB and facultative anaerobic, Cr-reducing bacteria isolated from nature and also the kinetics of Cr (VI) reduction in relation to sulfate and nitrate reduction under microaerobic conditions. The anaerobic sulfate-reducing bacteria (SRB) reduce Cr (VI) indirectly: by production of hydrogen sulfide, a strong reducing agent. Under specific experimental conditions, the cytochromes of SRB were found to reduce Cr (VI) directly. Supplemented substrates: sodium salts of acetate, formate, butyrate, or benzoate or with straw of the water plant cattail Typha latifolia L [25].

Sulfate reduction occurred in two stages: (1) slow, with no accumulation of hydrogen sulfide under high redox conditions, (2) rapid, with sulfide accumulation after 20 days when the redox potential had decreased and nitrate and Cr (VI) had been exhausted.

In the absence of sulfate reduction, direct Cr-reduction was accompanied by denitrification the process being initiated by the addition of natural substrate.

Genschow et al., 1996, studied the process of biological sulphate reduction in tannery wastewater in two stages anaerobically aiming to remove most of sulfate in the first stage of treatment followed by methane generation in the second stage. Sulfate removal was significantly influenced by the feed flow and pH which was found optimum at 7.0. Overall sulfate reduction achieved between 55 and 58% with the sulfate removal in the influent was 1180 mg/ l wherein the sulfate in the first stage was approximately 30%. But in the second stage, the desulfurization rate decreased with higher concentrations of sulfate in the influent [26].

Yun-guo et al. 2008, investigated the simultaneous removal of Cr(VI) and phenol in a consortium culture containing *Bacillus sp.* as the Cr(VI) reducer *Pseudomonas putida Migula* (CCTCC AB92019 as the phenol degrader. Phenol was used as the sole carbon source. *Bacillus sp*. utilized metabolites formed from phenol degradation by *Pseudomonas sp*. as energy source and electron donors for Cr(VI) reduction. With the phenol concentration of 150 mg/L and an initial Cr(VI) concentration of 15 mg/L, optimum Cr(VI) reduction of 83.67% was observed [27].

Yun-guo et al.2008, isolated four Chromium resistant bacteria from Cr-electroplating sludge which were identified as a member of *Bacillus* sp. The strain, YB-1 was found to resist 140mg/l of Cr.They also studied the living and freeze dried YB-1 biomass to remove Cr(VI) in pH 2.0, initial Cr concentration 60mg/l and The sorption capacity was found to be 45 mg/g and 28.21 mg/g for the biosorbent dose of just 0.01 g (dry mass) for both living and freeze-dried cells. They also established different isotherms for the biosorption process at 30^{o}C and kinetic models. One important finding is that reduction of Cr(VI) may take place while chromium is bound to the living cells [28].

Singh Rajesh et al., 2011, conducted experiments to investigate the chromium(VI), COD and sulphate removal efficiency in simulated amd real effluent (CETP) in a small scale bioreactor using a consortium of sulphate reducing bacteria. Maximum chromium(VI) and sulphate removal achieved was 96.0% and 82.0%, respectively at initial Cr(VI) concentration of 50 mg/1. The FTIR spectrum of the sulphate reducing bacterial consortium, as established by the authors indicated the existence of the sulphate ions which means sulfate reducing bacteria had used sulfate (SO_4^{2-}) during the growth phase and reduced it to sulphide (S^{2-}) using Cr(VI) as the electron donor and reduced it to Cr(III) [29].

2.2 Shortcomings of the available processes:

From the above study, it's evident that no system is simple and efficient enough for a complete treatment of tannery waste. Some of the above processes may not be economically feasible because of high costs and expertise required to implement and sustain the operation of such processes.
Conventional pretreatment methods followed by biological processes make it uneconomical. The S and Cr are chemically removed before heading it for microbial treatment processes.

If the process is too long, it becomes infeasible for treating huge amount of waste generated. Often nutrient supplements need to be added for biological processes.

A review of the technical literature indicates that many processes have been investigated for tannery wastewaters, ranging from the simple physico-chemical treatment to the advanced complicated biological treatment methods. Some industrial plants treat the Cr-rich wastewater by conventional aerobic or anaerobic treatment plants but are often not efficient enough and cost-effective. Biotechnological approaches to the abatement and remediation of toxic metal pollution consist of selectively using and enhancing the microbe mediated natural processes to treat particular wastes. The processes of interaction of microorganisms with the toxic metals enabling their removal/and recovery are biosorption, bioaccumulation and enzymatic reduction.

Tannery waste water contains exceptionally high concentration of Chromium. No Sulphate reducing bacteria can survive that high concentration. Hence in the first stage, the Cr(VI) is attempted to be brought down following which the SRBs can be employed to reduce sulphates and residual Cr(VI). This may be accompanied by COD and other pollutant removal in the industrial water treatment set-ups.

PROJECT OBJECTIVE:

- This contribution aims to find a suitable, efficient and cost-effective biological treatment of leather industry waste water.
- Establishing a consortium of microbes that can effectively survive and reduce high concentrations of Chromium(IV) and Sulfates

SPECIFIC OBJECTIVES:

- ✓ Isolation , screening and characterization of Cr(VI) resistant microbes from tannery waste
- ✓ Determining the maximum tolerance limit of Chromium and Sulphate
- ✓ Characterization of the waste water sample by parameters such as COD, BOD, TSS, TDS, TOC, TKN, SO_4^{2-} and Cr *etc*.
- ✓ Confirmatory test for the bacterial consortium for Cr removal
- ✓ Optimizing the process parameters in batch culture
- ✓ Treatment of wastewater in a fermenter
- ✓ Anaerobic post-treatment by a consortium of Sulphate reducing bacteria(SRBs)
- ✓ Final result and evaluation of the two stage tannery waste treatment

Chapter 3

MATERIAL & METHODS

3.1 Characteristics of wastewater:

All applied analyses were carried out according to "Standard Methods for Examination of Water and Wastewater" (APHA, 2002).

3.1.1 ***Color*****:** The observed color of the sample was deep green.

3.1.2 ***pH*****:** As measured by a digital pH meter, pH of the waste water was found to be 4.27.

3.1.3 ***Dissolve Oxygen (DO):*** DO was determined by a Dissolve Oxygenmeter (HACH, HQ_{10}) at 27^{o} C.

3.1.4 ***Biochemical oxygen demand (BOD*****):** BOD was experimentally determined by measuring the DO of a buffered and oxygenated water and sample diluted by the same and incubated at 27^{o} C for 3 days in BOD bottles.

Initial DO of the diluted sample: D_0

DO after 3 days of incubation: D_3

Initial DO of distilled water: C_0

DO after 3 days (blank): C_3

BOD at 3-day, 27^{o} C = $(D_0 - D_3)$* Dilution factor – $(C_0 - C_3)$ mg/l

3.1.5 ***Electrical Conductivity(EC):*** Electrical Conductivity was measured by the laboratory method of calculating conductivity at 25^{o}C by a Digital Conductivity Meter (EI Products, Model 621).

3.1.6 ***Total Dissolved Solids*****(*****TDS*****):** TDS was digitally measured by (EI, Model 661 E).

3.1.7 ***Total Organic Carbon (TOC)*****:** Inorganic carbon poses little to no threat. Total Organic Carbon in sample was determined instrumentally by TOC analyzer (SHIMADZU, TOC-VCPN).

3.1.8 ***Cr Concentration*****:** Chromium Sulphate is used as the tanning agent and hence the effluent after tanning contains high amount of Cr. The total Cr was measured by Atomic Absorption Spectroscopy (Perkin Elmerl, A-Analist200).

Cr(VI) was measured spectrophotometrically by Diphenyl Carbazide method which is nearly specific for Cr(VI). Adding Diphenyl Carbazide solution(Snell and Snell and Snell, 1959) (prepared by adding 250mg of Diphenyl carbazide to 50ml acetone and stored in a brown bottle) to pre-acidified samples develops a pink-purple color which can be measured with a UV-Vis spectrophotometer in 540 nm (JASCO, V-530).

3.1.9 ***Sulphate Concentration*****:** Sulphate estimation in the sample was carried out by $BaCl_2$ precipitation method. $BaCl_2$ reacts with SO_4^{2-} present in the sample in a buffered medium and forms $BaSO_4$ which unless let to precipitate, creates turbidity which was measured by a UV-Vis spectrophotometer (JASCO, V-530) at 420nm.

3.2 Sampling and Storage:

The waste water sample and solid chrome shavings after the tanning stage of leather processing were collected from Central Leather Research Institute (CLRI), Chennai, India in sterile conditions and stored at 4^o C to inhibit any further biological activity.

3.3 Isolation of microbes:

The wastewater contained a diverse flora of microbes. 15µl of freeze thawed sample was spread on an Lauria Bartini-Agar: Yeast extract 0.5%, Peptone 1%, Sodium Chloride (NaCl) 1%, Agar powder 1.5% and (pH 7.0 ± 0.2) plates and incubated at 30^o C for 72 hrs. A number of bacterial and fungal colonies appeared on the plates.

To separate the bacterial flora from fungal, each colony was further sub-cultured on LB-Agar plates containing antifungal agent "Fluconazole" to restrict fungal growth on it. The four pure bacterial isolate were named as B1, B2, B3 & B4.

(Figure 1: Isolated bacterium (B1) *Pseudomonas aeruginosa*)

(Figure 2: Isolated bacterium (B2) *Micrococcus yunnanensis*)

(Figure 3: Isolated bacterium:B4)

3.4 Enrichment and screening of the microbes:

The obtained microbial colonies were further sub-cultured to acclimatize them with high Cr concentration. The media for bacterial culture war supplemented with increasing amount of Chromium in form of $K_2Cr_2O_7$ typically starting with 20mg/l and gradually increasing it to 260mg/l by repeatedly sub-culturing. Individual microbial species was streaked separately and incubated for 72 hrs to screen out bacteria which can resist Cr at a high level and to determine the minimum inhibition concentration(MIC).

Further, it was needed to isolate microbes which can also grow in Sulphate containing medium. Hence Sulphate was added to media in form of Na_2SO_4 in very small quantities (10mg/l) along with the aforementioned Cr(VI) source and sub-cultured. Repeated sub-culturing was carried out to select as well as acclimatize the microbes to higher Cr and Sulphate levels.

3.5 Sub-culturing of pure culture for inocula preparation:

Isolated and acclimatized pure cultures were sub-cultured at an interval of every 15 days and used as inoculums for further studies.

3.6 Characterization of microorganisms:

Isolated microbial strains were characterized to identify the family and genus they belong to. The characterization was based on morphological, physiological and biochemical test and comparing the results with available data. Microbial characterization was done at *Microbial Type Culture Collection and Gene Bank (MTCC),* Chandigarh, India.

3.7 Consortia Study:

Microbial isolates as individual stains as well as in different combinations were tested in batch cultures for biomass growth and biodegradation of Cr(VI) and SO_4^{2-}. In 100ml media containing 150mg/l $K_2Cr_2O_7$ and 200mg/l Na_2SO_4, 30µl (0.3%) overnight grown culture was inoculated in strictly sterile conditions. The 7 combinations taken were, B1, B2, B4, B1+B2, B2+B4, B1+B4, B1+B2+B4. The flasks were incubated at 30^{o}C and 120 rpm for 96 hrs. Sampling was performed at every 4 hrs interval, biomass was separated out by centrifugation and the supernatant was analyzed for Cr(VI) and SO_4^{2-}. Reduction in Cr (VI) and SO_4^{2-} was measured also

spectrophotometrically using Diphenycarbazide method and $BaCl_2$ precipitation method respectively.

3.8 Biomass estimation:

Culture broth (1ml) was centrifuged (REMI, RM 12C micro centrifuge) at 8000rpm for 10 minutes to get the biomass precipitated. The pellet was washed with distilled water and centrifuged again. The obtained biomass was resupended in 1ml distilled water. The biomass was estimated by measuring Optical Density (OD) of biomass spectrophotometrically at 600 nm and comparing with a standard curve of known cell concentrations. This analysis includes all bacterial cells, dead and alive.

3.9 OPTIMIZATION OF PROCESS PARAMETERS:

As evident form the literature review and preliminary experiments carried out, the factors or conditions affecting the process are pH, Temperature, Agitation and initial Chromium concentrations. These parameters were optimized aiming to obtain the maximum growth of microorganism and also highest pollutant removal.

Classical design of experiment methods is too complex and not easy to use. A large number of experiments have to be carried out when number of parameters increases. The Taguchi method uses a special design of Orthogonal Arrays to evaluate the entire space of effecting parameters with a significantly smaller number of experiments.

In this study, the process parameters were optimized by Grey based Taguchi method using Minitab 14 software. An L9 orthogonal array with four columns and nine rows was used where the columns correspond to the factors specified in this study, and each column contains three levels (a total of 9 conditions) for the factors assigned to the column. The experimental layout of four parameters is given in Table 9. Taguchi recommends analyzing the means and S/N ratio using conceptual approach that involves plotting the effects and visually identifying the factors and values of that appear to be significant.

3.9.1 Design of Experiment by Taguchi Orthogonal Array Method:

For pH, the levels were varied as pH 5.0, 6.0 &7.0, The Temperature considered for optimization were 25^{o}C, 30^{o}C & 35^{o}C and agitation in form of Revolutions Per Minute (80, 100, & 120 rpm). The initial Cr concentration in form of K_2Cr2O_7 were taken as 150mg/l, 200mg/l & 400mg/l keeping Na_2SO_4 concentration constant at 300mg/l. The media used for the shake-flask was same LB broth and pH was adjusted with 0.1N HCL and 0.1N NaOH.

The Grey relational analysis based on grey system theory was used to solve the complicated interrelationship between the multiple responses effectively. In a Grey system, data pre-processing is the primary stage. Data pre-processing is a means of transferring the original values to a comparable sequence (the process of normalization) as the unit and range in one data sequence may differ significantly from other [34].

Steps for Taguchi analysis:

1. Normalizing the experimental results of biomass and Cr removal for all experimental runs.
2. Calculating the individual Grey relational Coefficient (GRC).
3. Calculating the overall Grey Relational Coefficient.
4. Performing statistical analysis for the input parameters with GRC that significantly affect the process.
5. Selecting the optimal levels of parameters.

The identification of the better performance for both biomass yield and Cr removal is “higher the better”.

In the analysis of Grey relation for “higher the better” response normalization, uses Equation 1.

Response normalization for “higher the better” condition:

$$X *_{ij} = \frac{x_{ij} - x_{min}}{x_{max} - x_{min}} \quad \text{..... equation 1}$$

Where x^{*}_{ij} is the normalized data and x_{ij} is the observed data.

After preprocessing the data, the individual Grey co-efficient of each normalized datum is given as equation 2,

$$\xi_{ij} = \frac{\Delta_{min} + \tau * \Delta_{max}}{\Delta_{ij} + \tau * \Delta_{max}}$$

…….……equation 2

Where Δ_{ij} was he j^{th} value in Δ_I different data series. Δ_{max} and Δ_{min} were the global maximum and minimum values in the different series respectively. The distinguished co-efficient τ lies between 0 to 1. The most preferred value if τ is 0.5 and same was considered here.

After calculating Individual Grey Coefficient, the overall grey Coefficient was calculated using equation 3.

$$\gamma = \frac{1}{n}\sum_{i=1}^{n} \xi_{ij}$$

……..equation 3

Where n is the no of response types, here n=2. The value of γ reflects the overall degree of standardized deviation of original data series from the reference data series.

With these values, the single he signal to noise ratio and relevant graphs were plotted in *Minitab 14* and the optimum values of parameters were determined.

3.10 Treatment in a bench-top fermenter:

A fermenter is to provide a controlled, contained and homogenous environment in which fermentation of bacterial, fungal cultures can be performed safely and practically to achieve particular objectives.

Laboratory scale production of the microorganisms and removal of both Cr and Sulphate was carried out in a 2litre capacity bench-top fermenter (IIC, Labeast Bio fermenter) maintaining the optimized parameter values (discussed in the following chapter) of pH 7.0, Temperature 35^{o}C, agitation 100 rpm and initial Cr concentration of 200mg/l and sulphate of 300mg/l for 72 hours. The fermenter was equipped with pH sensor, internal cooling and heating system, magnetic agitator, DO sensor, dosing bottles, air inlet port, sampling port *etc*. The fermenter vessel and all

other components were autoclaved prior to the assembling and fitting. The media was separately prepared and poured aseptically into the vessel.

Composition of the media used in the fermentation vessel is as follows (**Table 2**):

Component	**g/L**
Yeast extract	5.0
Peptone	10.0
Sodium Chloride	10.0
$K_2Cr_2O_7$	0.2
Na_2SO_4,	0.3

Overnight grown culture of both B1 and B2 was used as inoculum in 1:1 ratio of total 0.3% of fermentation media. Inoculum was charged into the vessel after running the fermenter with agitation and aeration for half an hour.

pH maintenance is very important in the cultivation process, where a little change in the pH will affect the physiochemical characteristics of the cells resulting in the shift in the metabolic pathway. pH adjustment was done by dosing acid or alkali whenever there is a shift in the pH using a fixed speed peristaltic pump. 1N HCl and 1N NaOH were used for the purpose.

Foaming was observed in the media after some time due to the amount of protein in the media as well as the protein produced during the process. To control the foam, n-Hectadecane was used in minimum amount.

Sampling was performed at an interval of every 8 hours and headed for estimation of biomass, Chromium and Sulphate removal.

(Figure 4: Fermenter set-up, *IIC, Labeast Bio fermenter*)

3.11 SRB Culture:

Sulphate Reducing Bacteria(SRB) exerts a great ecological, environmental and economic impact. SRB have found application in the bioremediation of toxic contaminants like biodegradation of toluene and xylene *etc.* SRB has been reported in the treatment of a variety of sulfate-containing industrial and mining effluents.

In the concerned study, the SRB is applied to reduce Sulphates as well as remaining Chromium from the first stage treatment of tannery effluent. In this context, a Sulphate Reducing Bacterial consortium of *Desulfovibrio desulfuricans*, *D. vulgaris* and *D. gigas* (Purchased from *Microbial Type Culture Collection and Gene Bank (MTCC)*, Chandigarh, India) were cultured in Postgate B medium, (*Postgate*,1984) [34] which is semi- specific for culturing SRBs. In 250 ml Erlenmeyer Flask, 100 ml Postgate B medium (pH 7.0) was prepared and autoclaved at 121^{o}C and 15lb pressure for 15 mins. All the three bacterial species were cultured in broth separately for the purpose of inocula preparation.

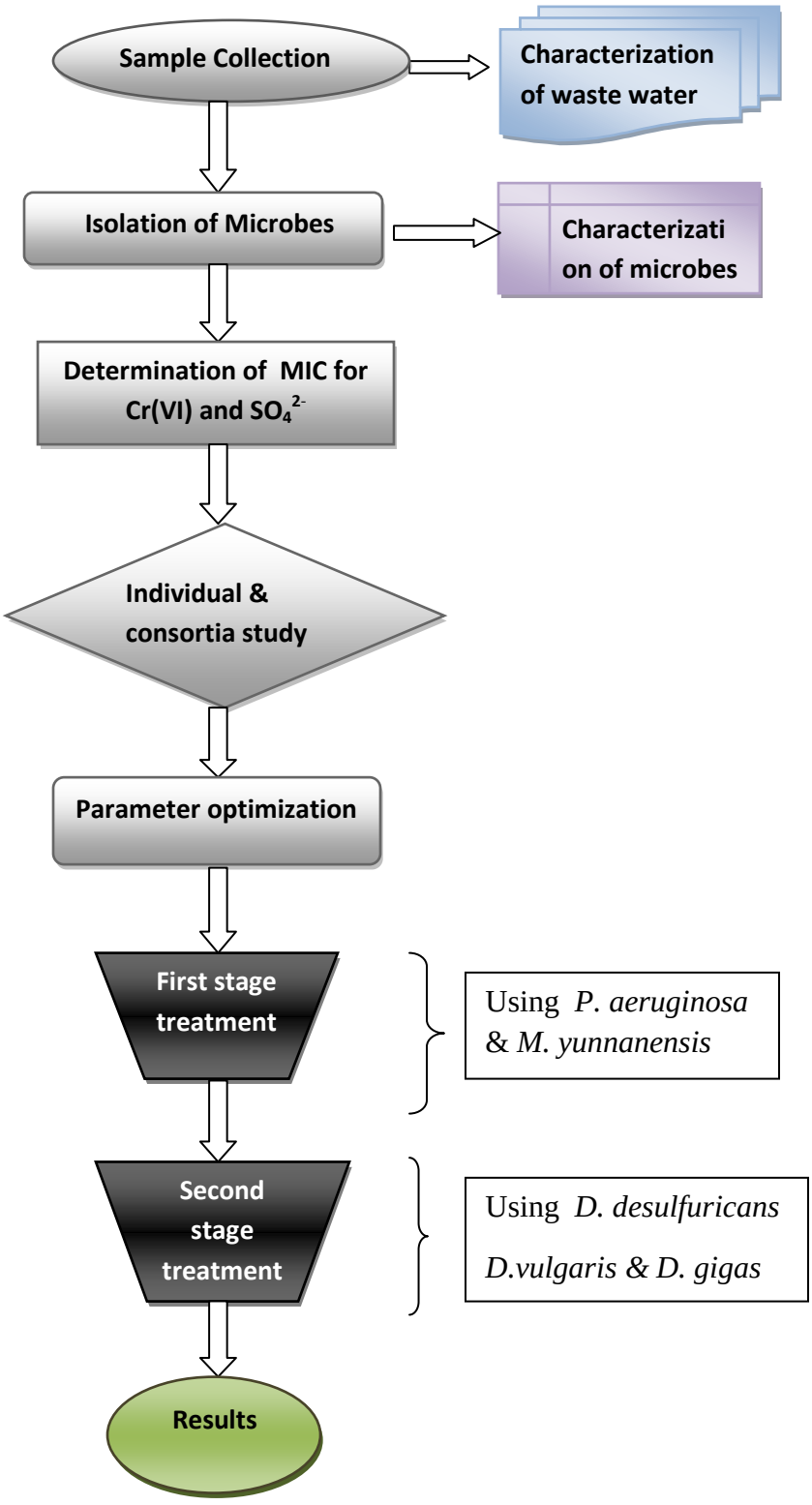
For the simulated media, undegraded Cr(VI) from the first stage treatment was supplemented in form of $K_2Cr_2O_7$ and 156 mg/l of Na_2SO_4. Subsequently, the aforementioned (consortium of SRB) microbes were inoculated (0.3%, 1:1:1 ratio) under strictly sterile conditions. The flask was incubated at 35^oC and 120 rpm for 5 days.

SRB culture medium (Postgate B) composition:

Component	**g/L**
Na_2SO_4	1.0
KH_2PO_4	0.5
NH_4Cl	2.0
$CaCl_2$	0.06
$FeSO_4$	0.005
Yeast extract	1.0
Sodium lactate	15 ml

Sampling was performed every 12 hours. The cell free extract was obtained by centrifuging 1 ml of culture medium at 8000 rpm and collecting the supernatant. Cr(VI) as well as SO_4^{2-} analyses were performed on the cell free broth as per the procedures already mentioned.

3.12 Project steps:



(Figure 5: Project flow diagram)

Chapter 4

RESULTS & DISCUSSION

4.1 Waste sample analysis:

The sample of tannery waste contained was analyzed for various water quality parameters to assess the degree of pollution in it. Table 3 contains the list of parameters, acceptable limits and observed values.

Table 3: List of parameters, permissible limits and observed values (US EPA)

Sl.no.	Pollutant or property	Acceptable limit	Sample Analysis
1	Color	Colorless	Dark green
2	pH	6.5-8.5	4.27
3	Dissolve oxygen(DO) (mg/l)	5	6.59
4	Biological Oxygen Demand(BOD) (mg/l)	40	3500
5	Chemical Oxygen Demand (COD) (mg/l)	140	4200
6	Total Dissolved Solid(TDS) (mg/l)	1000	44,550
7	Total Organic Carbon (TOC) (mg/l)	4	3,246
8	Conductivity (ms)	0.05	60.4
9	Sulphate (mg/l)	750	1900-2400
10	Sulphites (mg/l)	0.002	1-2
11	Chromium (mg/l)	0.05	1200-2500

- The color of the waste sample was imparted by the presence Chromium salt in it. however production of chromium(III) by reduction of chromium(VI) compounds gives chromium(III)

with some of the water ligands replaced with other species, e.g. $[Cr(H_2O)_4Cl_2]^+$. Such ions are green, and water displaces the other ligands very slowly indeed.

- pH of the sample was found to be 4.27. The acidic pH may be due to acidification of hides before tanning using weak acids like Formic acid and Sulphuric acid.
- The effluent sample exhibits a high BOD value indicating a high microbial population.
- High organic carbon content is left due to carbohydrates, proteins, oils from the raw hide. It's obvious to have very high Cr and sulphate concentrations in post tannery waste as these are the result of salts used in tanning process. The high organic content shows a greater scope of biological growth with less addition of nutrients.

4.2 Maximum Tolerance Limit:

Maximum Tolerance Limit (MTL) is the dose above which 99% of the population does not survive. Repeated sub-culturing of all the four bacterial isolates with increasing concentrations of Cr(IV) and SO_4^{2-} and B1 species was found to tolerate a maximum of 240 mg/l $K_2Cr_2O_7$ and 280mg/l Na_2SO_4.

Table 4: Microbes and Maximum Tolerance Limits

Isolate Name	$K_2Cr_2O_7$(mg/l)	Na_2SO_4(mg/l)
B1	240	280
B2	170	200
B4	170	200

With increasing heavy metal concentration in the medium, the microbes were observed to adjust to the gradual change in its environment, a process known as acclimatization or acclimatization. It occurs in a short period of time within the organism's lifetime. The microbes isolated and screened on nutrient plates with supplements of Cr and Sodium Sulphate shows acclimation to the altered environment. However, there are costs associated with acclimation and the growth rate is observed to decrease considerably for the bacteria. The degree or to which an organism is able to acclimate is estimated by it's phenotypic plasticity or the flexibility of the organism to change

certain traits within it. The mechanisms of metal toxicity are varied, but ultimately result in the denaturation and inactivation of enzymes, and in the disruption of cell organelles and membrane integrity. Cr tolerance is mainly a secondary outcome of metabolism due to metal sequestration as metal sulfides or enzymatic reduction of heavy metals within the cytoplasm. Many efflux mechanisms are associated with heavy metal tolerance.

4.3 CHARACTERIZATION STUDY:

The following morphological, physiological and biochemical tests were performed and on the basis of results, the microbial species were identified.

Table 5: Morphological Test results of bacterial strains

Tests	B1	B2
Colony morphology		
Configuration	Circular	Circular
Margin	Entire	Entire
Elevation	Flat	Raised
Surface	Smooth	Smooth
Texture	Moist	Moist
Pigment	Blue-Green	Yellow
Opacity	Opaque	Opaque
Gram's reaction		
Cell shape	Rods	Cocci
Spores(-)	-	-
Motility	+	-

'+' : positive; '-' : negative

Table 6: Physiological Test results of bacterial strains:

Growth at temperatures	B1	B2
4°C	-	+
10°C	+	+
25°C	+	+
30°C	+	+
37°C	+	+
42°C	+	+
55°C	-	-
Growth at pH		
pH 5.0	-	-
pH 6.0	+	+
pH 7.0	+	+
pH 8.0	+	+
pH 9.0	+	+
pH 10.0	-	-
pH 11.0	-	-
pH 12.0	-	-
Growth on NaCl (%)		
2.0	+	+
4.0	+	+
6.0	+	+
8.0	+	+
10.0	-	-
11.0	-	-
12.0	-	-

'+' : growth observed; '-' : no growth observed

Table 7: Biochemical Tests results of bacterial strains

Tests	B1	B2
Methyl red test	-	-
Vogesproskauer test	-	-
Citrate	+	-
Nitrate	+	-
Indole	-	-
Arganinedihydrolase	+	+
Gelatin hydrolysis	+	-
Starch hydrolysis	-	-
Esculine hydrolysis	-	-
Deoxyribonuclease	-	-
Urease	+	-
Catalase test	+	+
Oxidase test	+	-
Acid Production from		
Arabinose	+	-
Galactose	+	-
Mannitol	+	-
Xylose	+	-
Lactose	-	-
Dextrose	+	-

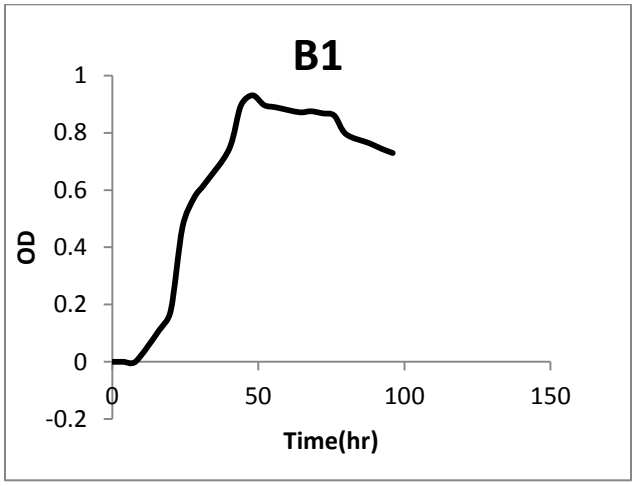
'+' : positive response; '-' : negative response

On the basis of above tests, the organisms have been identified as follows (Table 8):

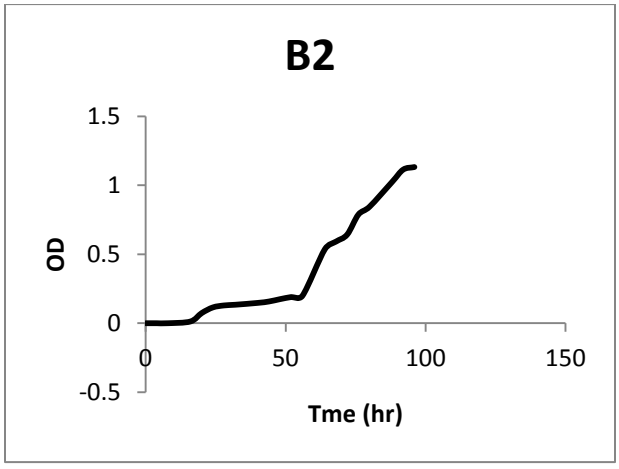
S. No.	Strain designation	Identity
1	B1	***Pseudomonas aeruginosa***
2	B2	***Micrococcus yunnanensis***

4.4 CONSORTIA STUDY:

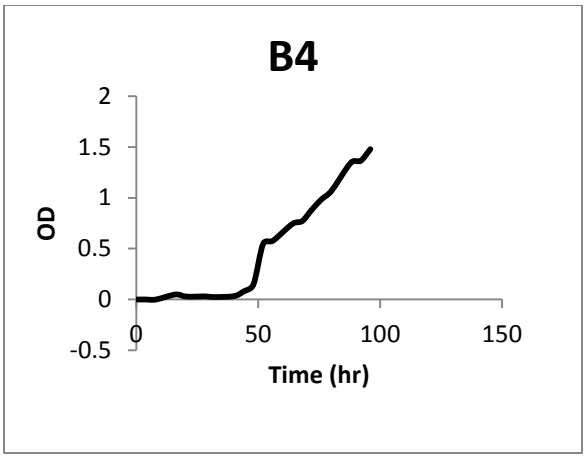
4.4.1 Growth rates: The objective was to establish the growth pattern for each set of microbe and find out the optimal growth with maximum removal of pollutants. Figure (6-12) represent the growth curve of microbes individual as well as in different consortia under the already mentioned conditions.



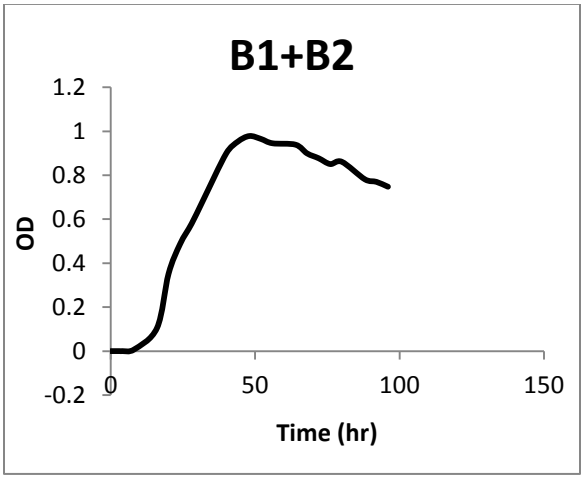
(Figure 6: Biomass growth curve for B1)



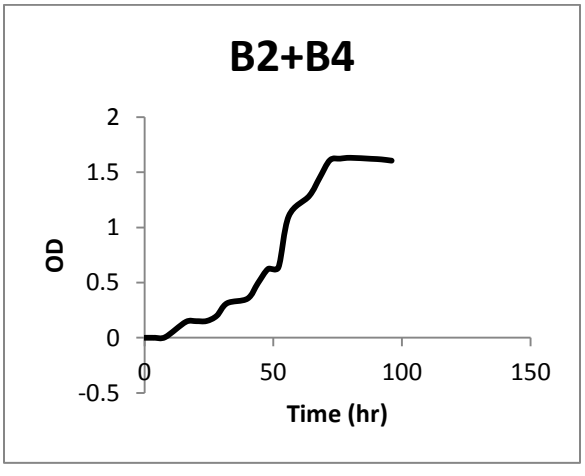
(Figure 7: Biomass growth curve for B2)



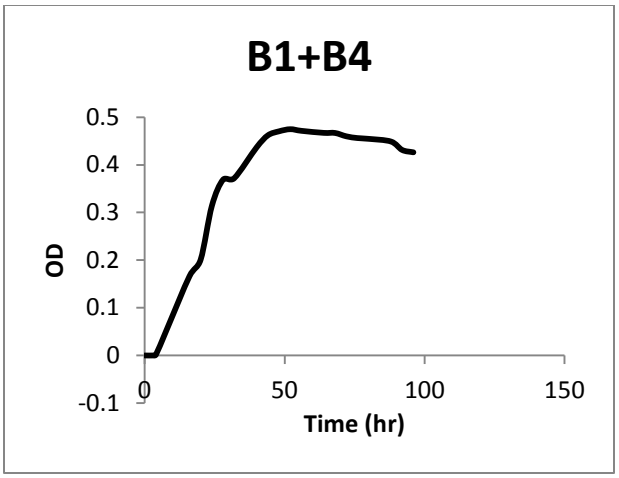
(Figure 8: Biomass growth curve for B4)



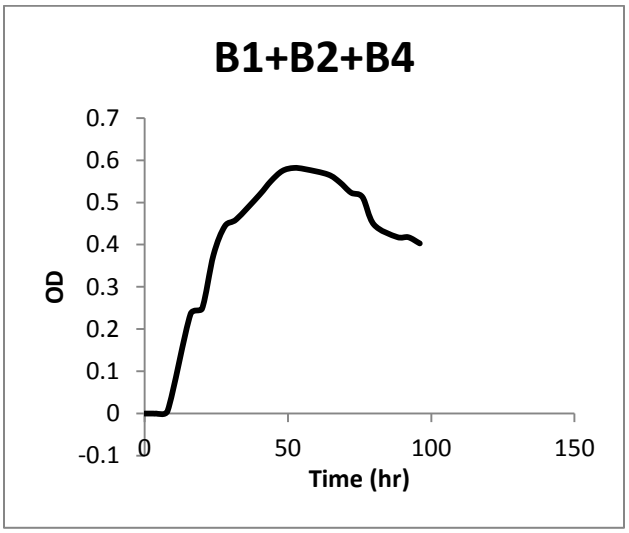
(Figure 9: Biomass growth curve for B1+B2)



(Figure 10: Biomass growth curve for B2+B4)



(Figure 11: Biomass growth curve for B1+B4)



(Figure 12: Biomass growth curve for B1+B2+B4)

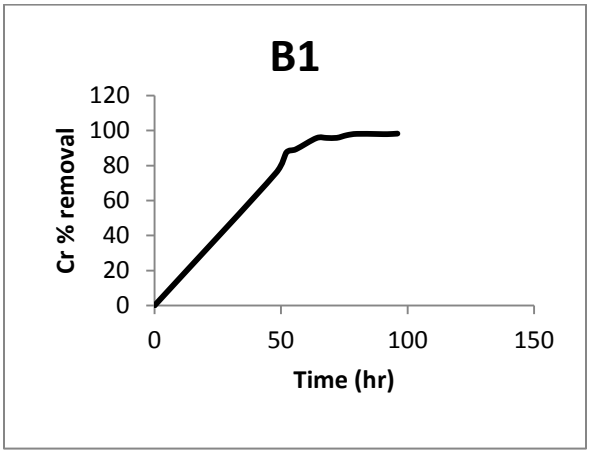
Observing the trend of Growth Curves all the individual isolates, it can be concluded that B1 is the fastest growing microbe which attains highest biomass in about 42 hours and remains in the stationary phase for some time and starts declining. Whereas, B2 had a stretched lag phase and were still in the exponential phase by the end of experiment. It attains more biomass growth than B1. B4 showed negligible growth up to about 44 hours and after that grows vigorously to attain the

maximum biomass concentration until the end of 96 hours. However, it was still in the growing phase.

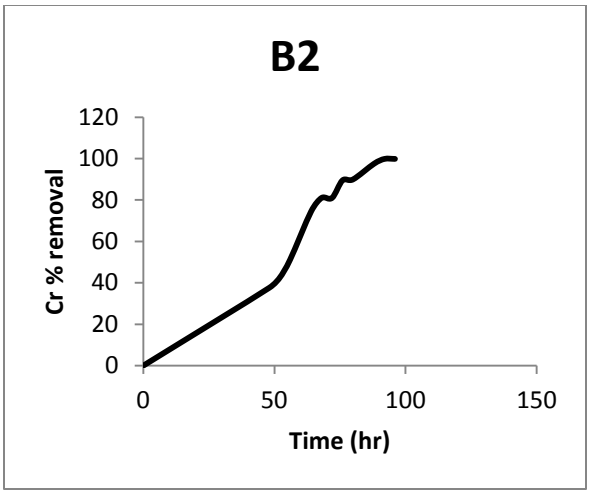
The mixed cultures containing B1 followed the similar pattern of growth and showed a high growth rate but quicker stationary phase. B1+B2 attains its maximum biomass at about 48 hours. But B2+B4 had a delayed lag phase and remained in the log phase even after 96 hours of culturing and the biomass growth was highest among all combinations. As evident from the graph (Figure 11, 12), the combinations B1+B2 and B1+B2+B3 had poor performance in terms of biomass growth.

4.4.2 Chromium(VI) removal:

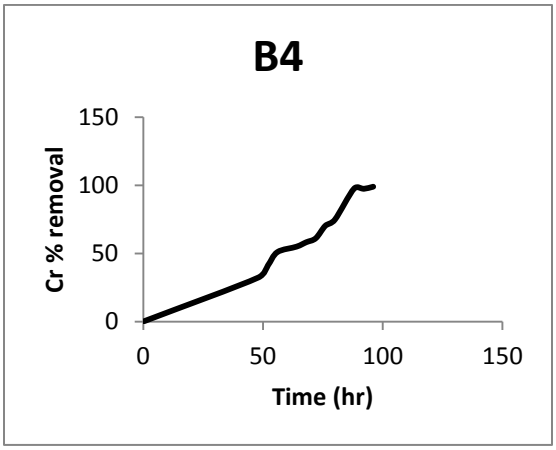
The major aim of this experimentation was to remove toxic form of Cr from the waste water and successfully a removal of 99.8% of Cr from culture broth was achieved. The graphs (Figure 13-19) show various patterns followed by different combination of microbes. Again B1 rapidly removed Cr up tp about 95% in just 64 hours. But maximum removal was achieved by B2 i.e. 99.85% in 92 hours. In this context, even the combination of B1 & B2 had a good efficiency. The consortium removed 95 % in 64 hours and achieved almost 100% removal at the end of 96 hours of experimental period.



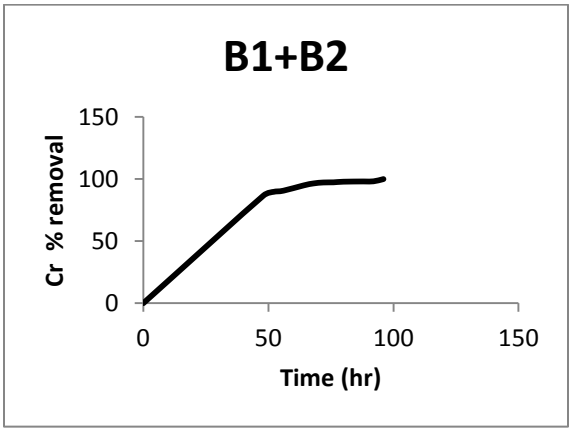
(Figure 13: Cr removal by B1)



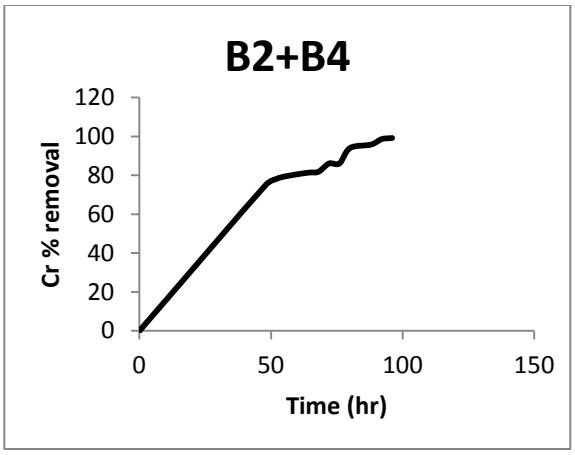
(Figure 14: Cr removal by B2)



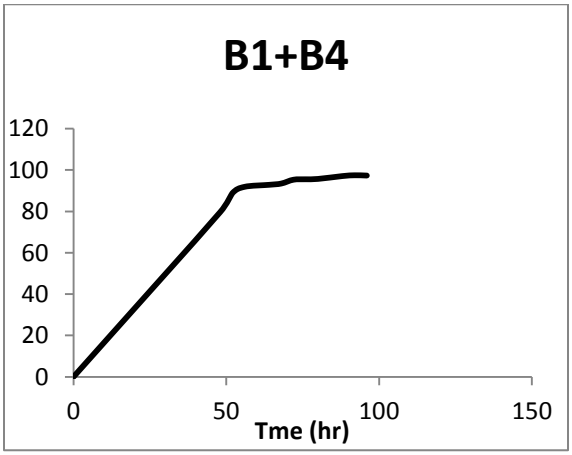
(Figure 15: Cr removal by B4)



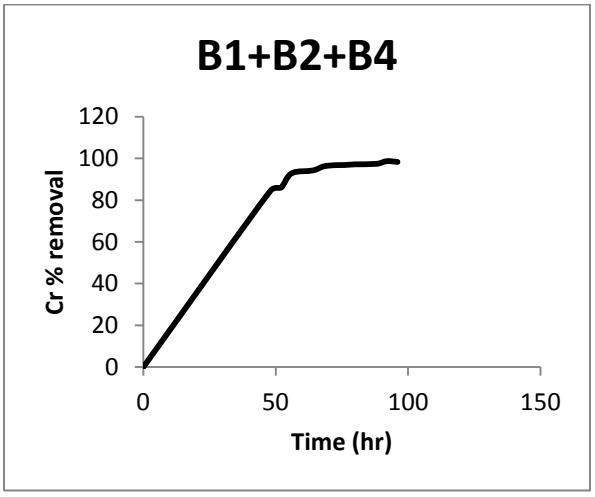
(Figure 16: Cr removal by B1+B2)



(Figure 17: Cr removal by B2+B4)



(Figure 18: Cr removal by B1 + B4)



(Figure 19: Cr removal by B1+B2+B4)

B4 bacteria were quite slow in remediating the pollutant but finally achieved 99% removal. Similarly other combinations such as B1+B4 and B1+B2+B4 also were capable of taking out almost 98% of Cr from the media. To summarize, every isolate obtained from tannery waste showed excellent Cr(VI) removal capacity. But the time taken by B1 and B1+B2 for the removal action was very less, which draws attention towards using these for bioremediation.

Among the isolates, none were identified as Sulphate Reducing Bacteria but individual studies showed that B1 and B2 degraded SO_4^{2-} in little quantities, B1 being more efficient. Hence from these test confirmatory experiments, the search was narrowed down to a consortium of B1+B2 which showed extended exponential phase, good biomass growth, rapid and efficient Cr(VI) and some sulphate removal and B1+B2 is the highest performing consortium.

Hence B1+B2 were selected for further parameter optimization.

4.5 Mechanism of bioremediation:

The high toxicity of chromium(VI) is due to its higher solubility, rapid permeability through cell membranes and subsequent interaction with intracellular macromolecules [30].

Cr(VI) is reduced to Cr(V) inside bacteria which is oxidized back to Cr(VI). Reactive oxygen species (ROS) are generated when Cr(VI) donates it's electron to molecular oxygen. In general, bacteria are found to employ several reductase enzymes against ROS.

P. aeruginosa has access to different resistance mechanisms against toxic metal stress due to induced over expression of varieties of proteins.

- Bacteria are known to express various stress proteins, such as heat shock proteins, starvation proteins and molecular chaperones *etc*. as a defensive response to a wide range of environmental stress conditions; such as heavy metals, oxidizing agents, suboptimal temperatures, starvation, different pH values.
- Some mechanisms for tolerating heavy metals are strategies such as exclusion by a permeability barrier, intra- and extra-cellular sequestration, active transport efflux pumps, enzymatic methods and a reduction in the sensitivity of targeted cellular organelles to metal ions [31].

- *P. aeruginosa* uses a plasmid-encoded chromate transporter (ChrA) for active export of chromium ions from the cytoplasm but this assigns resistance not removal of heavy metal [32].
- Chromate exposure may cause upregulation of genes encoding GSH synthetase and Glutathion-S-Transferase, GST, thioredoxin, enzymes and compounds which are responsible for combating heavy metal stress in *P. aeruginosa* by fighting ROS.
- The major composition of the outer cell membrane of Gram-negative bacteria is the anionic lipopolysaccharide layer, which has chelating abilities for metal ions and heavy metals are found to adsorb on them. Hence upregulation of genes coding the outer membrane components, particularly lipo-polysaccharides, will lead to an increase in heavy metal binding by allowing Cr(VI) to adsorb to its surface.

The microorganism, *P. aeruginosa* hss been found not to reduce Cr(VI) to Cr(III) [33]. Hence it may rely on one or several measures to remediate Cr(VI) stress.

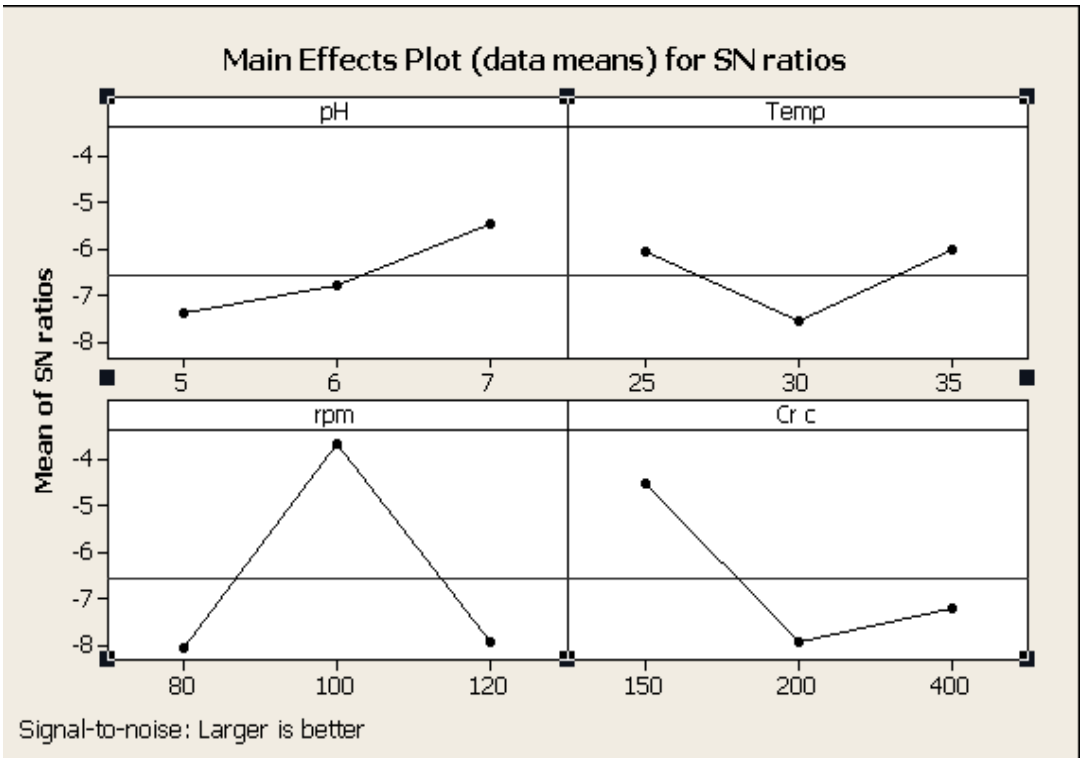
The other microbial species, *Micrococcus yunnanensis* may also employ any of these mechanisms to combat heavy metal stress.

4.6 Parameter Optimization:

Table 2 contains the different sets of experiments taken for optimizing process parameters as designed by Taguchi OA method, response and signal to noise ratios. As the selection criteria for both biomass and % Cr removal were higher the better, the highest s/n ratio was the most optimal value.

Table 9: DoE by Taguchi OA method and analysis (*Minitab 14*)

SET	pH	Temp (°C)	Agitatior (rpm)	Initial Cr conc (mg/l)	Response	SNRA1	PSNRA1
Set1	5	25	80	150	0.47902	-6.39289	0.0000
Set2	5	30	100	200	0.45085	-6.91941	
Set3	5	35	120	400	0.35787	-8.92548	
Set 4	6	25	100	400	0.62708	-4.05352	
Set 5	6	30	120	150	0.43989	-7.13314	
Set 6	6	35	80	200	0.34729	-9.18617	
Set 7	7	25	120	200	0.40952	-7.75447	
Set 8	7	30	80	400	0.37055	-8.623	
Set 9	7	35	100	150	1	0	



(Figure 20: data means for S/N ratios by Taguchi DoE, *Minitab 14*)

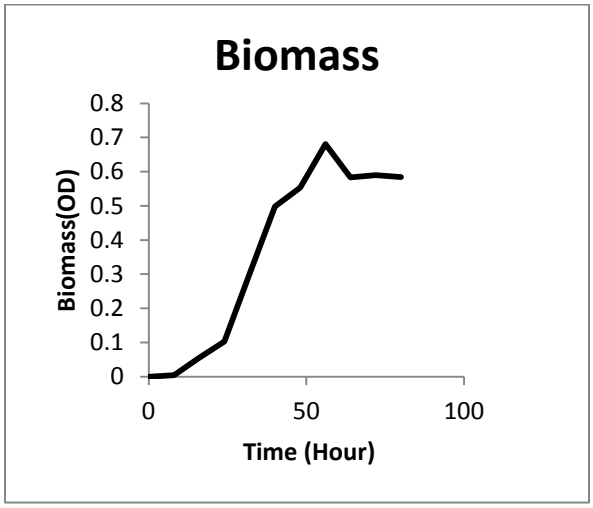
From the above plot of S/N ratios, it can be easily concluded that the optimum set of parameters for the most efficient process is pH 7.0 temperature 35^{o}C, rpm 100 and initial Cr concentration 150mg/l. the values were crosschecked to confirm the results.

4.7 Fermentation Process:

In the controlled environment of optimized parameters, the laboratory scale treatment of pollutant was carried out and samples were analyzed. Both the microbes were aerobic and hence the first stage of treatment was aerobic.

4.7.1 Biomass:

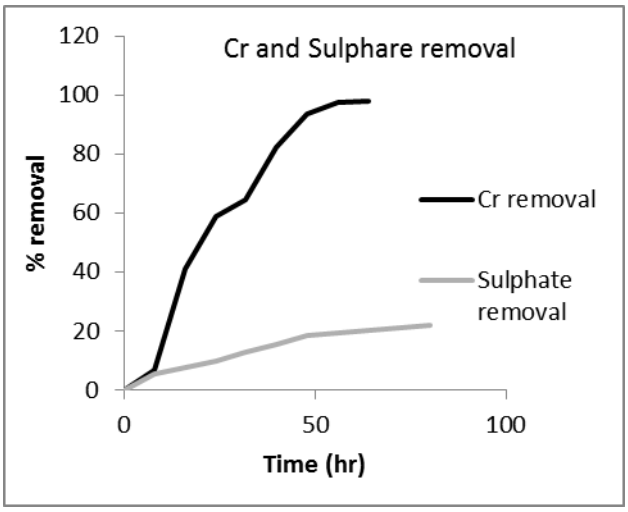
(Figure 21) represents the growth of biomass inside the fermenter. The highest growth was found at 48 hours followed by a stationary phase.



(Figure 21: Biomass B1+B2 growth profile in fermenter)

4.7.2 Cr removal:

(Figure 22) represents the percentage Cr removal by the consortia with time. It's evident from the graph that about 97.5 % Cr have been removed by 48th hour and the peak i.e. 98% is reached in 64 hours of fermenter run.



(Figure 22: Cr and sulphate removal curve in the fermenter process)

The results with optimized parameters are much faster and better. Only as less as in 58 hours, over 97% Cr removal is achieved. Hence the consortium is efficient in removal of pollutant from Tannery waste.

(Table 10: Comparison of Cr(VI) removal ability of different microbes)

Name of the m/o	Initial Cr Conc(mg/l)	% Removal	Reference
B. sphaericus	20	62	A. Pal *et al*., 2004
Bacillus sp.	15	84.4	Yun-guo *et al*., 2008
SRB consortium	50	97	Sahinkaya *et al.*, 2012
SRB consortium	50	96.0	S. Rajesh *et* al., 2011
P. aeruginosa	108	98.12	Present study
M. yunnanensis	108	99.8	Present study

4.8 Removal of Cr(VI) and Sulphate in second stage:

The removal obtained at 35^{o}C and pH 7.0 in shake flask culture was 97.5% for Cr(VI) and 54.9% for SO_4^{2-}

Sulfate Reducing Bacteria (SRB) are chemoorganotrophic, obligate anaerobic bacteria that use sulfate as a terminal electron acceptor for respiration during which the sulfate is reduced to hydrogen sulfide (eq 1 & 2). $SO^{2-}{}_4 + COD \rightarrow H_2S + CO_2$ ……..(1)

$Me^{n+} + n/2S^{2-} \rightarrow MeS\downarrow$ ……..…..**.....**(2)

Indirect reduction of Cr(VI) takes place were Cr(VI) acts as a terminal electron acceptor along with generation of H_2S. SRB is often found to reduce Cr(VI) directly in their cytochromes.

.

(**Table 11**: Overall removal of pollutants due to the treatment)

Pollutant name	**Chromium(VI)**	**Sulphates(SO_4^{2-})**
Initial Conc(mg/l)	150	200
After first stage(mg/l)	4.02	156
% removal	97.3	22
After second stage(mg/l)	0.1	70.2
% removal	97.5	54.9
Overall removal	99.9	63.9

Hence in the post second stage anaerobic treatment, the final concentration of Cr(VI) is in agreement with the permissible discharge limits for industrial effluent. The final sulphate concentration is also way below the acceptable limits

Chapter 5

CONCLUSION

The aim of this study was to obtain and establish a bacterial culture with potential utility in the bioremediation of tannery waste waters polluted with heavy metal Cr and sulfate.

- The tannery effluent analyzed was found to contain excessively high amount of Cr, Sulphate and organic carbon content which can be hazardous if returned back to the environment untreated.

- Acclimation of the cultures to high metal and sulphate concentrations was carried out in the pure isolates. Bacterial culture (*Pseudomonas aeruginosa),* displaying 240mg/l Cr and 280mg/l sulfate resistance has been achieved. Maximum tolerance limit for *Micrococcus yunnanensis* was 170mg/l and 200 mg/l for Cr and sulphates respectively.

- The consortium of *Pseudomonas aeruginosa* & *Micrococcus yunnanensis* had a considerable growth and pollutant removal capacity especially for Cr(VI).

- For a more efficient removal of pollutants, the optimal parameters were pH 7, temperature 35^{o}C, rpm 100 and Cr concentration 150 mg/l as calculated by Taguchi OA approach. *Pseudomonas aeruginosa* + *Micrococcus yunnanensis* consortium had better biomass growth and bioremediation of Cr as established by the experiments carried out.

- Laboratory scale fermenter study focused on Cr removal, maintaining the optimized parameters showed 97.5 % removal of Cr in just 48 hours and the highest of which is 98% achieved in 56 hours. Apart from Cr, about 20% of sulphate was also removed

- The Cr removal stage is followed by treatment with a consortium of Sulphate removing Bacteria(SRB) of *Desulfovibrio desulfuricans, D. vulgaris* and *D. gigas* separately in batch . Sulphate was found to reduce upto 70.2 mg/l from an initial concentration of 156 mg/l (54.9%)

accompanied by Cr(VI) reduction upto 0.1mg/l (97.5%). Thus the acceptable discharge limit for both the considered pollutants has been met.

As an ending note, a two stage biological treatment process is being proposed. The first stage is solely focused on Chromium removal up to very low levels which can be efficiently achieved by a consortium of aerobic *Pseudomonas aeruginosa* & *Micrococcus yunnanensis* followed by an anaerobic post treatment with a Sulphate reducing bacterial consortium to achieve both residual Chromium as well as sulphate removal. Hence the biological treatment of tannery waste for Cr and SO_4^{2-} has been established.

Suggestions for future work:

- Mechanism of Cr(VI) removal by *Micrococcus yunnanensis* is to be studied.
- Parameter optimization for the second stage SRB treatment
- Research to find efficiency of these microbes in sequestering other heavy metals
- Checking the reduction of COD load after SRB treatment
- Tracking the other pollution parameters *viz*, BOD, TOC, TDS, Sulphides, TKN *etc.*
- Efficiency of immobilized microbes in the process is needed to be examined

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